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| <p>(54) Title: IMPROVED NON-NUCLEOTIDE-BASED LINKER REAGENTS FOR OLIGOMERS</p> <p>(57) Abstract</p> <p>Novel non-nucleotide reagents for incorporation into oligomers are provided which optionally have a chirally pure skeleton and non-nucleotide reagents are provided which may be coupled into a non-nucleotide/nucleotide polymer. These reagents optionally have a chirally pure skeleton and may include a linker arm to which a detectable label or cross-linking agent may be conjugated.</p> | | |

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IMPROVED NON-NUCLEOTIDE-BASED LINKER REAGENTS
FOR OLIGOMERS

BACKGROUND OF THE INVENTION

Non-nucleotide based linking reagents for labelling oligonucleotides have a distinct advantage over nucleotide based reagents in that they do not adversely affect the normal base pairing between an oligonucleotide and a target nucleic acid and allow attachment at any location within the oligonucleotide or on its termini. Several non-nucleotide based linking reagents have already been described for use in labelling standard oligodeoxyribonucleotides, i.e. having a phosphodiester backbone (Lyle J. Arnold, Jr., et al., "Non-Nucleotide Linking Reagents for Nucleotide Probes", PCT WO 8902439 assigned to Gen-Probe, Inc.). However, those non-nucleotide reagents were of mixed chirality. Those reagents are coupled into the phosphodiester backbone of an oligonucleotide by chemical synthesis as disclosed therein.

Recently, other non-nucleotide based linking reagents have been described. For example, one of the reagents referred to specifically in the Gen-Probe patent application has also been described by Paul S. Nelson et al., (Nucleic Acids Res., 1989, vol. 17, p. 7179). The synthesis of polyamide-oligonucleotide probes for use in attaching nonisotopic labels has also been described by J. Haralambidis et al. (Nucleic Acids Res., 1990, vol. 18, p.501).

SUMMARY OF THE INVENTION

One aspect of the present invention provides non-nucleotide reagents which are suitable for preparing nucleotide/non-nucleotide polymers and which remain chirally pure when incorporated in a nucleotide/non-nucleotide polymer. These non-nucleotide reagents comprise: a non-nucleotide monomeric

unit which has an enantiomerically (or chirally) pure non-nucleotide skeleton and, connected to the skeleton, has a ligand moiety and first and second coupling groups. The first coupling group is capable of coupling the skeleton to a first additional monomeric unit, while the second coupling group remains inactivated so as to be substantially incapable of coupling, but the second coupling group can thereafter be activated under non-adverse conditions to couple the skeleton to a second additional monomeric unit, wherein said nucleotide/non-nucleotide polymer comprises at least one nucleotide monomeric unit.

In another aspect, the present invention provides non-nucleotide reagents which are useful in preparing nucleotide/non-nucleotide polymers which have alkyl- or aryl- phosphonate diester linkages between monomeric units. The non-nucleotide reagent comprises a non-nucleotide monomeric unit which has a non-nucleotide skeleton and connected to the skeleton has a ligand moiety and first and second coupling groups, wherein the first coupling group is capable of forming an alkyl- or aryl-phosphonate linkage between the skeleton and a first additional monomeric unit while the second coupling group remains inactivated so as to be substantially incapable of coupling, but which second coupling group can thereafter be activated under non-adverse conditions to couple the skeleton to a second additional monomeric unit, wherein said nucleotide/non-nucleotide polymer comprises at least one nucleotide monomeric unit.

According to one preferred aspect of the present invention, novel non-nucleotide based reagents are provided which are useful in preparing nucleotide/non-nucleotide polymers which have intermonomeric unit alkyl- or aryl- phosphonate linkages. These non-nucleotide reagents comprise enantiomerically pure non-nucleotide monomeric units which have an enantiomerically (or chirally) pure non-nucleotide skeleton and connected to the skeleton have a ligand moiety and first and second coupling

groups wherein the first coupling group is capable of forming an alkyl- or aryl- phosphonate linkage between the enantiomerically pure skeleton and a first additional monomeric unit, while the second coupling group remains inactivated so as to be substantially incapable of coupling, but which second coupling group can thereafter be activated under non-adverse conditions to couple the chirally specific skeleton to a second additional monomeric unit, wherein said nucleotide/non-nucleotide polymer comprises at least one nucleotide monomeric unit.

The ligand moiety may comprise a linker-arm group which may participate in conjugation reactions upon its activation or deprotection, a detectable chemical moiety or label or a side arm to which a detectable chemical moiety or label has been attached prior to initiating synthesis of the polymer. Suitable chemical moieties include detectable labels, chelators, catalysts, nucleolytic moieties, drug carriers, hormone receptors, substances which enhance oligomer uptake by cells, haptens for hormone receptors, and the like. Suitable chemical moieties include psoralen and analogs thereof, acridines and analogs thereof, porphyrins and porphyrin analogs, cyclic chelators and the like.

Definitions

As used herein, the following terms have the following meanings, unless expressly stated to the contrary:

The term "nucleotide" refers to a subunit of a nucleic acid consisting of a phosphate group, a 5 carbon sugar and a nitrogen containing base. In RNA the 5 carbon sugar is ribose. In DNA, it is a 2-deoxyribose. The term also includes analogs of such subunits.

The term "nucleotide multimer" refers to a chain of nucleotides linked by phosphodiester bonds, or analogs thereof.

An "oligonucleotide" is a nucleotide multimer generally about 10 to about 100 nucleotides in length, but which may be greater than 100 nucleotides in length. They are usually considered to be synthesized from nucleotide monomers, but may also be obtained by enzymatic means.

An "deoxyribooligonucleotide" is an oligonucleotide consisting of deoxyribonucleotide monomers.

A "polynucleotide" refers to a nucleotide multimer generally about 100 nucleotides or more in length. These are usually of biological origin or are obtained by enzymatic means.

A "nucleotide multimer probe" is a nucleotide multimer having a nucleotide sequence complementary with a target nucleotide sequence contained within a second nucleotide multimer, usually a polynucleotide. Usually the probe is selected to be perfectly complementary to the corresponding base in the target sequence. However, in some cases it may be adequate or even desirable that one or more nucleotides in the probe not be complementary to the corresponding base in the target sequence.

A "non-nucleotide monomeric unit" refers to a monomeric unit which does not significantly participate in hybridization of a polymer. Such monomeric units must not, for example, participate in any significant hydrogen bonding with a nucleotide, and would exclude monomeric units having as a component, one of the 5 nucleotide bases or analogs thereof.

A "nucleotide/non-nucleotide polymer" refers to a polymer comprised of nucleotide and non-nucleotide monomeric units.

An "oligonucleotide/non-nucleotide multimer" is a multimer generally of synthetic origin having less than 100 nucleotides, but which may contain in excess of 200 nucleotides and which contains one or more non-nucleotide monomeric units.

A "monomeric unit" refers to a unit of either a nucleotide reagent or a non-nucleotide reagent of the present invention, which the reagent contributes to a polymer.

A "hybrid" is the complex formed between two nucleotide multimers by Watson-Crick base pairings between the complementary bases.

The term "oligomer" refers to oligonucleotides, nonionic oligonucleoside alkyl- and aryl-phosphonate analogs, phosphorothioate analogs of oligonucleotides, phosphoramidate analogs of oligonucleotides, neutral phosphate ester oligonucleotide analogs such as phosphotriesters, and other oligonucleotide analogs and modified oligonucleotides, and also includes nucleotide/non-nucleotide polymers. The term also includes nucleotide/non-nucleotide polymers wherein one or more of the phosphorous group linkages between monomeric units has been replaced by a non-phosphorous group linkage such as a formacetal linkage or a carbamate linkage.

The term "alkyl- or aryl-phosphonate oligomer" refers to nucleotide oligomers (or nucleotide/non-nucleotide polymers) having internucleoside (or intermonomer) phosphorus group linkages wherein at least one alkyl- or aryl- phosphonate linkage replaces a phosphodiester linkage.

The term "methylphosphonate oligomer" (or "MP-oligomer") refers to nucleotide oligomers (or nucleotide/non-nucleotide polymer) having internucleoside (or intermonomer) phosphorus group linkages wherein at least one methylphosphonate internucleoside linkage replaces a phosphate diester internucleoside linkage.

The term "nucleoside" includes a nucleosidyl unit and is used interchangeably therewith.

In some of the various oligomer sequences listed herein "p" in, e.g., as in ApA represents a phosphate diester linkage, and "P" in, e.g., as in CpG represents a methylphosphonate

linkage. Certain other sequences are depicted without the use of p or P to indicate the type of phosphorus diester linkage. In such occurrences, A as in ATC indicates a phosphate diester linkage between the 3'-carbon of A and the 5' carbon of T, whereas A, ATC or ATC indicates a methylphosphonate linkage between the 3'-carbon of A and the 5'-carbon of T or T.

The term "non-adverse conditions" describes conditions (of reaction or synthesis) which do not substantially adversely affect the polymer skeleton and its sugar, base, linker-arm and label components, nor the monomeric reagents. One skilled in the art can readily identify functionalities, coupling methods, deprotection procedures and cleavage conditions which meet these criteria.

The term "deblocking conditions" describes the conditions used to remove the blocking (or protecting) group from the 5'-OH group on a ribose or deoxyribose group.

The term "deprotecting conditions" describes the conditions used to remove the protecting groups from the nucleoside bases.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A, 1B and 1C depict the formulas of non-nucleotide reagents of the present invention having Fmoc-protected linker arms.

Figure 2 depicts a synthetic scheme for preparing non-nucleotide reagents of the present invention having C2, C4 and C6 linker arms.

Figure 3 depicts a synthetic scheme for preparing non-nucleotide reagents of the present invention having C8, C10 and C12 linker arms.

Figure 4 depicts a synthetic scheme for a psoralen reagent which may form a psoralen conjugate with the linker arm of one of the non-nucleotide reagents of the present invention.

DETAILED DESCRIPTION OF THE INVENTION

According to the present invention, we have invented certain novel non-nucleotide based linking reagents which are especially useful in incorporating ligands such as cross-linking agents in oligomers, especially alkyl- or aryl- phosphonate oligomers. These reagents may be coupled into an oligomer using the automated coupling chemistry used for coupling the nucleotide based phosphoramidite monomers. The resulting modified oligomers, including alkyl- or aryl- phosphonate oligomers, contain nucleophilic primary amines through which a variety of secondary compounds may be attached by standard aqueous chemistries already known in the art. Examples of secondary compounds include intercalators, alkylators, photoactive and reactive moieties such as psoralens, chelating agents, etc. We believe that by applying such chemistries, we will be able to increase the potency of methylphosphonate oligomers as therapeutic agents.

In a preferred aspect of the present invention, the non-nucleotide based linking reagents are prepared in a chirally pure form. Moreover, these non-nucleotide reagents remain chirally pure when incorporated in oligomers (non-nucleotide/nucleotide polymers). This advantage may be critical when it is desired or desirable to direct a label to a particular location when the oligomer is hybridized to a corresponding target nucleic acid. In a particularly preferred aspect, the hydrocarbon skeleton of these reagents comprises a reduction product of threonine. Since the four enantiomers of threonine are commercially available, non-nucleotide reagents having a chirally pure skeletons derived from any one of the four stereoisomers of threonine may be prepared.

The choice of threonine as the starting material to supply the chirally pure skeleton for some of these reagents has

additional advantages. First, a three carbon skeleton is available for insertion into the phosphorus backbone of the oligomer, which closely resembles the three carbon spacing of traditional deoxyribose groups. Second, reduced threonine has a primary hydroxyl and a secondary hydroxyl which enables the subsequent protection, deprotection, blocking, deblocking steps and derivatization steps to proceed in improved yields.

According to one aspect of the present invention, we have functionalized these non-nucleotide reagents in such a way that an alkyl- or aryl- phosphonate diester linkage results when these non-nucleotide reagents are inserted into the phosphorus backbone of the oligomer. Such reagents couple to nucleotides or other non-nucleotide reagents in high yield. Furthermore, we have demonstrated that these non-nucleotide reagents can be used for the attachment of psoralen analogs to the oligomers. These psoralen analogs are attached to a non-nucleotide reagent using a novel chemistry (See Example 13).

GENERAL PROPERTIES OF NON-NUCLEOTIDE REAGENTS

Thus, in general, the present invention provides a non-nucleotide reagent, with a non-nucleotide monomeric unit which can be coupled synthetically with specific nucleotide monomeric units from nucleotide reagents, to produce a defined sequence polymer which is comprised of nucleotide and non-nucleotide monomeric units. Said non-nucleotide reagent also possesses a ligand which may comprise a linker-arm moiety which may participate in conjugation reactions once the linker-arm has been deprotected, or may comprise a side-arm to which a useful desired chemical moiety has been attached prior to incorporating the non-nucleotide reagent in the polymer. In general, the techniques for linking moieties to the linker arm may be similar to the techniques for linking labels to groups on proteins. However, modifications of such techniques may be required. Examples of

useful chemistries include the reaction of alkylamines with active esters, active imines, arylhalides, or isothiocyanates, and the reaction of thiols with maleimides, haloacetyls, etc. (for further potential techniques see G.M. Means and R.E. Feeney, "Chemical Modification of Proteins", Holden-Day Inc., 1971; R.E. Feeney, Int. J. Peptide Protein Res., Vol. 29, 1987, p 145-161). Suitable protecting groups which can be used to protect the linker arm functional group during formation of a polymer are also similar to those used in protein chemistry (see for example, "The Peptides: Analysis and Synthesis, Biology," Vol. 3, ed. E. Gross and J. Meienhofer, Academic Press, 1971). Due to the chemical nature of the non-nucleotide reagent, it may be placed at any desired position within the nucleotide monomer sequence. This makes it possible to design a wide variety of properties into polymers which contain nucleotide monomers. These include:

- (1) attachment of specific chemical moieties at any desired location within the polymer, such moieties can include (but are not limited to) detectable labels, intercalating agents, chelators, drugs, hormones, proteins, peptides, haptens, radical generators, nucleolytic agents, proteolytic agents, catalysts, receptor binding substances, and other binding substances of biological interest, and agents which modify DNA transport across a biological barrier (such as a membrane), and substances which alter solubility of a nucleotide multimer. This means that it is possible to position such labels and intercalating agents adjacent to any desired nucleotide;
- (2) the ability to immobilize the defined sequence to a solid support employing its linker-arm for conjunction to a chemical moiety of said support in order to construct, for example, nucleotide affinity supports;
- (3) the ability to attach multiple chemical moieties to the polymer through linker-arms by incorporating multiple non-nucleotide monomeric units into the polymers;
- (4) the ability to construct polymers which differ from naturally occurring polynucleotides in

that they have altered activities with proteins and enzymes which act on polynucleotides. For example, the placement of the non-nucleotide monomeric unit on the 3' terminus of an otherwise pure polynucleotide imparts resistance to degradation by snake venom phosphodiesterase. Such non-nucleotide monomeric units may create specific cleavage sites for other nucleases; (5) the ability to construct hybridization probes by interspersing hybridizable nucleotide and non-nucleotide monomeric units. For example, a mixed block synthesis of nucleotide and non-nucleotide monomers can be produced, whereby a defined sequence of nucleotide monomers are synthesized followed by a stretch of the one or more non-nucleotide monomeric units followed by second block of defined sequence nucleotide monomers; (6) the ability to construct synthetic probes which simultaneously detect target nucleotide multimers which differ by one or more base pairs. This is accomplished by using the non-nucleotide reagent described herein to replace the nucleotides in the probe with non-nucleotide monomeric units at sites where differences occur in the nucleotide sequence of the various target nucleotide multimers.

In a preferred form of the invention, labelled oligomers are constructed with a defined sequence comprised of nucleotide and non-nucleotide monomers. In another preferred form of the invention, the non-nucleotide monomeric units are used to connect two or more defined sequence nucleotide multimers, and the non-nucleotide monomeric units are chemically labelled for use in cross-linking reactions.

In yet another preferred embodiment, the non-nucleotide reagent is constructed in a manner to permit it to be added in a step-wise fashion to produce a mixed nucleotide/non-nucleotide polymer employing one of the current DNA synthesis methods. Such nucleotide and non-nucleotide reagents normally add in a step-wise fashion to attach their corresponding monomeric units to a

growing oligomer chain which is covalently immobilized to a solid support. Typically, the first nucleotide is attached to the support through a cleavable ester linkage prior to the initiation of synthesis. Step-wise extension of the oligomer chain is normally carried out in the 3' to 5' direction. For standard DNA and RNA synthesis methods, see for example, "Synthesis and Applications of DNA and RNA" ed. S.A. Narang, Academic Press, 1987, and M.J. Gait, "Oligonucleotide Synthesis", IRL Press, Wash. D.C. U.S.A., 1984. When synthesis is complete, the polymer is cleaved from the support by hydrolyzing the ester linkage mentioned above and the nucleotide originally attached to the support becomes the 3' terminus of the resulting Oligomer. By analogy, an alternative way to introduce a non-nucleotide monomeric unit is to similarly attach it to a DNA synthesis support prior to initiation of DNA synthesis. In a preferred embodiment the non-nucleotide monomeric unit is attached to a DNA synthesis support through an ester linkage formed using the free alcohol form of the non-nucleotide monomer.

Accordingly, the present invention provides a non-nucleotide reagent for preparing polymers which contain a mixture of nucleotide and non-nucleotide monomeric units. Said non-nucleotide monomers additionally may contain one or more protected linker-arms or one or more linker-arms conjugated to a desired chemical moiety such as a label, a cross-linking agent or an intercalating agent.

Such a non-nucleotide monomer additionally possesses two coupling groups so as to permit its step-wise inclusion into a polymer of nucleotide and non-nucleotide monomeric units. A first one of said coupling groups has the property that it can couple efficiently to the terminus of a growing chain of monomeric units. The second of said coupling groups is capable of further extending, in a step-wise fashion, the growing chain of mixed nucleotide and non-nucleotide monomers. This requires

that the second coupling group be inactivated while the first coupling group is coupling, so as not to substantially couple at that time, but can thereafter be activated so as to then couple the non-nucleotide monomeric unit. The "inactivation" is preferably accomplished with a blocking group on the second coupling group, which can be removed to "activate" the second coupling group. However, it is within the scope of the invention that such "inactivation" and "activation" might be accomplished simply by changing reaction conditions (e.g., pH, temperature, altering the concentration of some other component in the reaction system) with second coupling groups of a suitable chemical structure, which also lend themselves to inactivation and activation by such techniques. Said coupling groups permit the adjacent attachment of either nucleotide or non-nucleotide monomeric units. In a preferred embodiment said coupling groups operate through coupling and deblocking and deprotection steps which are compatible with one of the standard DNA synthesis methods.

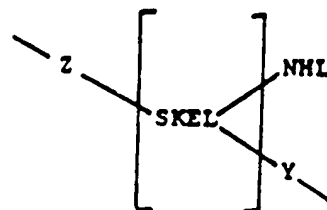
Such methods require that synthesis occur unidirectionally and that all coupling cleavage and deblocking or deprotection steps occur under "non-adverse" conditions, that is they do not substantially adversely affect the polymer skeleton and its sugar, base, linker-arm and label components nor the monomeric reagents. One skilled in the art can readily identify functionalities, coupling methods, deblocking and deprotection procedures, and cleavage conditions which meet these criteria (see, for example, the Gait reference, supra).

The non-nucleotide monomer preferably has a skeleton, to the ends of which the coupling groups are linked. The skeleton is preferably an acyclic one to twenty atom chain, and preferably an acyclic hydrocarbon chain of from one to twenty carbon atoms.

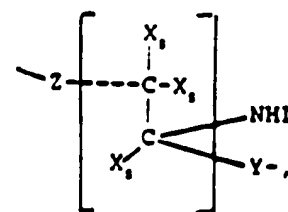
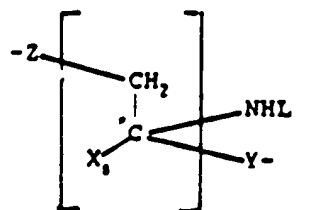
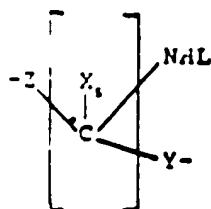
PREFERRED NON-NUCLEOTIDE REAGENTS

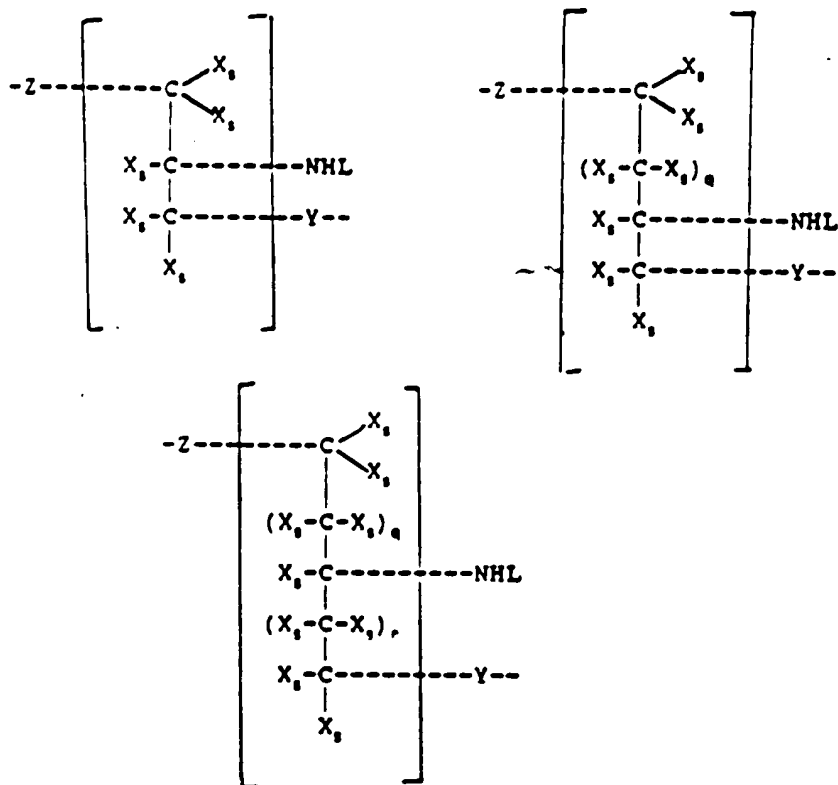
Preferred non-nucleotide reagents comprise non-nucleotide monomeric units in which the skeleton has a backbone of up to about 10 carbon atoms in which said backbone comprises at least one asymmetric carbon which remains chirally pure upon being coupled into a nucleotide/non-nucleotide polymer. Skeletons having backbones of about three carbons are preferred, in part, because such backbones resemble the three-carbon spacing of deoxyribose groups.

One preferred aspect of the present invention is directed to chirally pure non-nucleotide reagents which when incorporated in an oligomer comprise a chirally pure non-nucleotide monomeric unit of the formula:



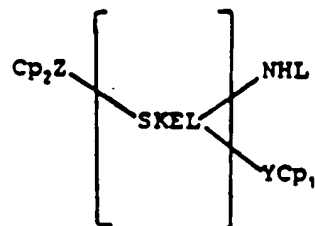
wherein SKEL comprises a chirally pure non-nucleotide skeleton of from about 1 to about 20 carbon atoms, wherein -NHL, Y and Z are covalently linked to a carbon atom of SKEL, L is a ligand, Y is -CH₂-, -O-, -S- or -NH- and Z is -O-, -S- or -NH-. Preferably SKEL further comprises a backbone of about 1 to about 10 carbon atoms separating Y and Z. Examples of non-nucleotide monomeric units incorporating these preferred SKEL groups include:





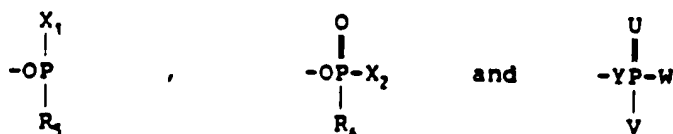
wherein the X_1 groups are independently selected from hydrogen or alkyl and may be the same or different, and q and r are independently selected integers from 0 to 10.

Thus, in one embodiment, these preferred non-nucleotide reagents may be represented by the general formula:



wherein ---YCp_1 is a first coupling group, ---ZCp_2 is a blocked second coupling group, wherein L , Y and Z are as defined above and

(a) the first coupling group, ---YCp_1 , is selected from:



wherein X_1 is halogen or substituted amino; X_2 is halogen, amino, or substituted amino, or O' ; R_3 is alkyl, optionally substituted alkoxy or optionally substituted aryloxy; and R_4 is alkyl, optionally substituted alkoxy or optionally substituted aryloxy, or if X_2 is O' , optionally hydrogen; U is oxygen, sulfur or imino, W is alkyl, aryl, alkoxy, aryloxy, alkylthio, arylthio, S' , O' , amino or substituted amino, and V is alkoxy, alkylthio, amino or substituted amino.

(b) blocked second coupling group, $-ZCp_2$, wherein Cp_2 is a blocking group cleavable under deblocking conditions to recover the second coupling group $-X'$ wherein Z is $-O-$, $-NH-$ or $-S-$.

Since preferred are non-nucleotide reagents which are capable of forming alkyl- or aryl-phosphonate, and in particular methylphosphonate, diester linkages between monomeric units, especially preferred non-nucleotide reagents include those wherein the first coupling group, $-YCp_1$, is selected from

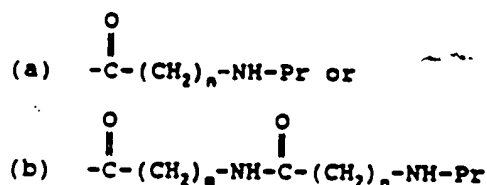


wherein X_1 is chloro or secondary amino and R_3 is alkyl; X_2 is substituted amino, halogen or O' and R_4 is alkyl.

The ligand moiety, L is preferably selected from a functional moiety or from a protected linking arm which can be deprotected under non adverse conditions so as to be capable of then linking with a functional moiety (under non-adverse conditions).

In one preferred aspect of the present invention, L comprises a protecting group, Pr , or protected linker arm which can be deprotected under non-adverse conditions so as to be capable of then linking with a functional moiety, including a

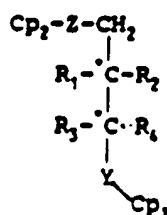
cross linking agent such as psoralen, or a drug carrier molecule. Preferred linker arms include those having one of the following formulas:



wherein n and m are independently integers between 1 and 15, preferably between 1 and 5, and Pr is a protecting group removable under non-adverse conditions.

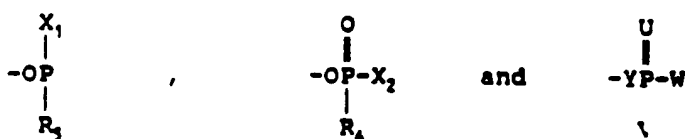
One group of particularly preferred non-nucleotide reagents has a skeleton derived from the amino acid threonine. These preferred reagents comprise a 3-carbon backbone having two asymmetric carbons, each of which remains chirally pure when incorporated in a nucleotide/non-nucleotide polymer. In addition, these reagents having threonine-derived backbones advantageously have a primary hydroxyl and a secondary hydroxyl, which due to their differing reactivities allow selectivity and high yields in the subsequent protection, deprotection, blocking, deblocking and derivatization steps. In one preferred embodiment of the present invention, the first coupling group is associated with the secondary hydroxyl group and the second coupling group is associated with the primary hydroxyl.

Thus, according to an especially preferred aspect of the present invention, the threonine-based non-nucleotide reagents have the following formula:



wherein 'C denotes an asymmetric carbon which is chirally pure, and wherein one of R_1 and R_2 is hydrogen and the other is $-NH-L$ where L is a ligand moiety as hereinafter defined; one of R_3 and R_4 is hydrogen and the other is lower alkyl of about 1 to about 10 carbon atoms, $-Y-Cp_1$ is a first coupling group, and $-ZCp_2$ is a blocked second coupling group, wherein:

(a) The first coupling group, $-YCp_1$, wherein Y is $-CH_2-$, $-S-$, $-NH-$, or $-O-$ is selected from



wherein X_1 is halogen or substituted amino; X_2 is halogen, amino, or substituted amino, or O' ; R_3 is alkyl, optionally substituted alkoxy or optionally substituted aryloxy; and R_4 is alkyl, optionally substituted alkoxy or optionally substituted aryloxy, or if X_2 is O' , optionally hydrogen; U is oxygen or sulfur, W is alkyl, aryl, alkoxy, alkylthio, aryloxy, arylthio, O' , S' , amino or substituted amino; and V is alkoxy, alkylthio, amino or substituted amino; and

(b) blocked second coupling group, $-ZCp_2$, wherein Cp_2 is a blocking group cleavable under deblocking conditions to recover the second coupling group $-ZH$ wherein Z is $-O-$, $-NH-$ or $-S-$.

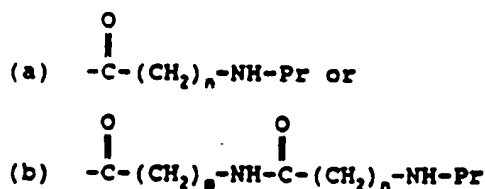
The ligand moiety, L is preferably selected from a functional moiety or from a protected linking arm which can be deprotected under non-adverse conditions so as to be capable of then linking with a functional moiety (under non-adverse conditions).

Since non-nucleotide reagents which are capable of forming alkyl- or aryl-phosphonate, and in particular methylphosphonate, diester linkages between monomeric units, are preferred especially preferred non-nucleotide reagents include those wherein the first coupling group, $-YCP_1$, is selected from



wherein X_1 is chloro or secondary amino and R_3 is alkyl; X_2 is substituted amino, halogen or O^- and R_4 is alkyl.

In one preferred aspect of the present invention, L comprises a protecting group, Pr, or a protected linker arm which can be deprotected under non-adverse conditions so as to be capable of then linking with a functional moiety, including a cross linking agent such as psoralen, or a drug carrier molecule. Preferred linker arms include those having one of the following formulas:



wherein n and m are independently integers between 1 and 15, preferably between 1 and 5, and Pr is a protecting group removable under non-adverse conditions.

Suitable protecting groups, Pr, include 9-fluorenylmethoxycarbonyl ("Fmoc"), trifluoroacetyl, phenoxyacetyl and the like. See, e.g., Chapter 7 of Greene, Theodore W., "Protective Groups in Organic Synthesis", John Wiley & Son, New York, 1981. These linker arms may be conveniently prepared according to the

reaction schemes outlined in Figures 2 and 3 and described the Examples herein.

UTILITY

These non-nucleotide reagents are useful in preparing oligomers having ligand moieties conjugated to the oligomer without adversely affecting the normal base pairing associated with hybridization to a target nucleic acid. These ligand moieties may comprise functional groups or protected linker arms which may later (after synthesis of the oligonucleotide) be deprotected and react with a labelling reagent to give a linker arm - labelling reagent complex. Functional groups of particular utility may include detectable labels, agents which react with a target nucleic acid such as cross-linking agents, agents which cleave the target nucleic acid, agents which increase the uptake of oligomers into cells or skin and agents which slow the excretion of oligomers from the body.

In certain instances, where these non-nucleotide reagents are incorporated into oligomers having alkyl- or aryl-phosphonate linkages between monomeric units, it may be advantageous to incorporate nucleotide monomeric units having modified ribosyl moieties. The incorporation of nucleotide units having 2'-O-alkyl-, in particular 2'-O-methyl, ribosyl moieties into alkyl- or aryl-phosphonate oligomers advantageously may improve hybridization of the oligomer to its complimentary target sequence.

To assist in understanding the present invention, the following examples follow, which include the results of a series of experiments. The following examples relating to this invention are illustrative and should not, of course, be construed as specifically limiting the invention. Moreover, such variations of the invention, now known or later developed, which would be within the purview of one skilled in the art are to be

considered to fall within the scope of the present invention hereinafter claimed.

EXAMPLES

EXAMPLE 1

REDUCTION OF L-THREONINE METHYL ESTER

L-Threonine methyl ester (purchased from Sigma) was reduced according to the procedure of Stanfield et al. (J. Org. Chem. (1981), 46, 4799): in a 500 ml three necked flask, 5 g of L-threonine methyl ester and 200 ml dry THF were mixed and 150 ml of 1 M solution of LiAlH_4 was added dropwise with stirring while under argon. The reaction mixture was then warmed up to the boiling temperature of THF and refluxed under argon overnight. The completion of the reaction was monitored by TLC on Silica Gel which was visualized with ninhydrin. The reaction mixture was cooled to 5-10° C and quenched with dropwise addition of 0.25 M NaOH (100 ml). The mixture was evaporated to remove over 90% of THF and the residue was diluted with 100 ml of dimethylformamide which facilitates the filtration. The mixture was then filtered through a Whatman #1 paper using aspirator vacuum. The filtrate was evaporated to dryness and the residue was purified on a flash Silica Gel column. The column was packed with dichloromethane and the product was eluted with 50% methanol in dichloromethane.

EXAMPLE 2

SYNTHESIS OF 4-N-(9-FLUORENYLMETHOXYCARBONYL)-

4-AMINO-n-BUTYRIC ACID

Fmoc-aminobutyric acid (for C4 linker arm) was prepared according to the following procedure. (Note: other Fmoc-aminocarboxylic acids are commercially available. For example, Fmoc-aminocaproic acid (for C6 linker arm) and Fmoc-glycine (for

C2 linker arm) are commercially available from Bachem, Inc., Torrance, California).

A mixture of 1.8 g 4-aminobutyric acid and 1.24 g sodium hydrogen carbonate in 35 ml water/acetone (50:50) was prepared and 5 g Fmoc-succinimidyl carbonate (N-Fluorenylmethyl-succinimidylcarbonate) (Bachem) was added. The reaction mixture was stirred overnight at room temperature. The pH of the reaction mixture was adjusted to 2 by 1N HCl and the solvent was removed under reduced pressure and the residue was dissolved in 20 ml ethanol and filtered. The filtrate was evaporated to dryness and the residue was taken up in dichloromethane and filtered to give 4.8 g of pure product.

¹H NMR in DMSO-d₆, 1.61 (CH₂), 2.22 (CH₂), 3.01 (CH₂-N), 4.32 (CH₂-C=O), 4.22 (NH), 7.25-7.95 (8 aromatic protons).

EXAMPLE 3

BLOCKING OF THE AMINE MOIETY OF REDUCED L-THREONINE

The amine moiety of the reduced L-threonine was coupled with a 9-fluorenylmethoxycarbonyl ("Fmoc") group using with a procedure similar to the Fmoc-aminobutyric acid preparation described above. After the overnight reaction, adjustment of the pH was not necessary. The solvent was removed and the residue was dissolved in 40 ml dichloromethane and extracted with water (2 x 50 ml). The organic phase was then dried and purified on a flash Silica Gel column. The product was eluted with 2% methanol in dichloromethane to give 3.85 g of the product.

¹H NMR 1.20 (CH₃), 2.85 (NH), 3.26 (CH), 3.48 (CH), 3.72 (OH), 7.3-7.9 (8 aromatic protons).

EXAMPLE 4

PREPARATION OF FMOC-BLOCKED LINKER ARMS:

FMOC-GLYCYLAMIDO-CAPROIC ACID (C8), FMOC-4-AMINOBUTRYLAMIDO-CAPROIC ACID (C10) AND FMOC-CAPROXYLAMIDO-CAPROIC ACID (C12)

Fmoc-glycine, Fmoc-4-aminobutyric acid and Fmoc-aminocaproic acid were coupled to the aminocaproic acid in order to synthesize the above-identified C8, C10 and C12 linker arm. The desired Fmoc-amino acid (17 mmol) was dried with co-evaporation with dry pyridine (3 x 30 ml). The dried material was then dissolved in 30 ml of dry dimethylformamide and 30 ml dry tetrahydrofuran was added. The solution was cooled to 0°C and 1 equivalent (17 mmol) of N,N-diisopropylethylamine was added. While stirring, 1 equivalent of trimethylacetyl chloride was added dropwise at 0°C and stirred for 45 min. 1.2 equivalent of dry aminocaproic acid was then added and the reaction mixture was warmed up to room temperature and stirred overnight. The progress of the reaction was monitored by TLC. After the completion, the solvents were evaporated under reduced pressure. The residue was reconstituted with 50 ml water and the pH was adjusted to 2 by 1N HCl. The mixture was extracted with 100 ml of ethyl acetate and the organic phase was washed with 20 ml of water and dried (MgSO₄). The mixture was then filtered and the solvent was evaporated under reduced pressure to a volume of about 40 ml. Hexane was added dropwise to this solution until cloudiness and cleared by heating. The product was then crystallized overnight.

C8 ¹H NMR in DMSO-d₆, 1.30 (CH₂), 1.39 (CH₂), 1.48 (CH₂), 2.20 (CH₂-N), 3.06 (CH₂ of FMOC), 3.58 (CH₂-COOH), 4.24 (2NH), 4.34 (CH of FMOC and CH₂ of Glycine), 7.3-7.9 (8-Aromatic protons).

C10 ¹H NMR in DMSO-d₆, 1.30-1.70 (5CH₂'s), 2.07 (CH₂), 2.20 (CH-N), 3.0-3.1 (CH₂-COOH and CH₂ of FMOC), 4.26 (2NH), 4.31 (CH of FMOC), 7.3-7.9 (8-Aromatic protons).

C12 ¹H NMR in DMSO-d₆, 1.2-1.5 (6CH₂'s), 2.00 (CH₂-N), 2.18 (CH₂-N), 2.9-3.0 (2CH₂-C=O), 4.23 (2NH), 4.31 (CH₂ of FMOC), 7.3-7.9 (8-Aromatic protons).

EXAMPLE 5COUPLING OF REDUCED L-THREONINE TO LINKER ARMS

The desired linker arm (11 mmol), which was made according to Examples 2 or 4 above (Fmoc-glycine (C2), Fmoc-4-aminobutyric acid (C4), Fmoc-caproic (C6), Fmoc-glycylamidocaproic acid (C8), Fmoc-4-aminobutyrylamidocaproic acid (C10), and Fmoc-aminocaproylamidocaproic acid (C12)), was dried with co-evaporation with pyridine (3 x 20 ml). The dry residue was dissolved in 40 ml of a mixture of anhydrous dimethylformamide and anhydrous tetrahydrofuran (1:1). The solution was cooled in an ice bath and 1 equivalent of diisopropylethylamine was added. While stirring, 1.1 equivalent of trimethylacetyl chloride was added dropwise and stirred for 45 min at 0°C. A solution of 1.5 equivalent of reduced L-threonine (Example 1 above) was added and the reaction mixture was allowed to warm to room temperature and stirred for one hour. The progress of the reaction was monitored by TLC on Silica Gel which was developed by $\text{CH}_2\text{Cl}_2/\text{CH}_3\text{OH}/\text{CH}_3\text{COOH}$ (10:1:0.1) solvent system. After the completion of the reaction, the solvent was removed under reduced pressure and the residue was mixed with 50 ml ethyl acetate. The water soluble materials were removed by extraction with 40 ml saturated sodium bicarbonate. The organic phase was washed with 20 ml of water and dried (MgSO_4). The product was crystallized from ethyl acetate.

C2 Linker ^1H NMR in DMSO-d_6 , 1.03 (CH_3 of reduced L-threonine), 3.35 (OH), 3.3-3.45 (2CH), 3.91 (NH), 4.27 (other NH), 4.31 (OH), 4.34 (CH_2), 4.63 (CH_2 and CH of Fmoc), 7.3-7.9 (8-Aromatic protons).

C4 Linker ^1H NMR in DMSO-d_6 , 1.03 (CH_3 of reduced L-threonine), 1.62 (CH_2), 2.14 (CH_2), 2.91 (CH), 2.97 (CH_2), 3.3-3.5 (2CH), 3.63 (OH), 3.84 (OH), 4.23 (CH), 4.33 (CH and CH_2 of Fmoc), 4.60 (NH), 6.32 (NH), 7.3-7.9 (8-Aromatic protons).

C6 Linker ^1H NMR in DMSO- d_6 , 1.03 (CH_3 of reduced L-threonine), 1.3-1.7 (3 CH_2 's), 2.52 ($\text{CH}_2\text{-N}$), 3.12 (CH-C=O), 3.8-3.9 (2 OH), 4.1-4.2 (2CH), 4.41 (CH_2 of Fmoc), 5.22 (NH), 6.48 (NH), 7.3-7.9 (8-Aromatic protons).

C8 Linker ^1H NMR in DMSO- d_6 major proton signals are as follows: 1.01 (CH_3 of reduced L-threonine), 1.22-1.52 (3 CH_2 of caproate), 3.62 and 3.84 (2 OH), 5.35 (NH), 6.18 (NH), 7.3-7.9 (8-Aromatic protons).

C10 Linker ^1H NMR in DMSO- d_6 , 1.02 (CH_3 of reduced L-threonine), 1.3-1.50 (4 CH_2 's), 3.64 (OH), 3.82 (OH), 4.64 (NH), 6.33 (NH), 6.62 (NH), 7.3-7.9 (8-Aromatic protons).

C12 Linker ^1H NMR in DMSO- d_6 , major proton signals for identification 1.01 (CH_3 of reduced L-threonine), 1.30-1.50 (6 CH_2 's), 3.63 (OH), 3.82 (OH), 4.62 (NH), 6.31 (NH), 6.63 (NH), 7.3-7.9 (8-Aromatic protons).

EXAMPLE 6

DIMETHOXY TRITYLATION OF THE PRIMARY HYDROXYL MOIETY OF THE NON-NUCLEOTIDE REAGENT

The desired non-nucleotide reagent (6 mmol), which was made according to Examples 3 and 5 above, was dried by co-evaporation with dry pyridine and dissolved in 15 ml of dry pyridine. A solution of 2.2 g of dimethoxytrityl chloride in 20 ml of CH_2Cl_2 /pyridine (1:1) was added dropwise with stirring. The reaction continued at room temperature for 45 min. The progress of the reaction was monitored by TLC. After the completion of the reaction it was quenched by the addition of 2 ml methanol which was stirred for 10 min. The solvents were removed under reduced pressure and the residue was dissolved in 50 ml of dichloromethane and extracted with saturated sodium hydrogen carbonate (2 x 50 ml) followed by water (30 ml). The organic phase was dried (MgSO_4) and filtered. After the evaporation of the solvent, the residue was purified with a flash

column chromatography. The product was eluted with 2% methanol in dichloromethane containing 0.5% triethylamine.

C0 Linker ^1H NMR, CDCl_3 , 1.18 (CH_3 of reduced L-threonine), 1.63 (CH), 2.83 (NH), 3.77 (2 CH_2 of DMT), 3.82 (CH_2 of FMOC), 5.48 (CH_2 -O-DMT), 6.82-7.90 (21 aromatic protons).

C2 Linker ^1H NMR, CDCl_3 , 1.18 (CH_3 of reduced L-threonine), 3.78 (2 CH_2 's of DMT), 4.35 (CH_2 -O-DMT), 5.98 (NH) 6.80-7.78 (21 aromatic protons).

C4 Linker ^1H NMR, CDCl_3 , major signals 1.18 (CH_3 of reduced L-threonine), 1.83 (CH_2), 2.28 (CH_2), 3.74 (2 CH_2 of DMT), 4.21 (OH), 4.38 (CH_2 of FMOC), 5.22 and 6.42 (2 NH), 6.80-7.65 (21 aromatic protons).

C6 Linker ^1H NMR, CDCl_3 , major peaks 1.12 (CH_3 of reduced L-threonine), 1.3-1.6 (3 CH_2 's), 3.75 (2 CH_2 of DMT), 4.38 (CH_2 of FMOC), 6.80-7.90 (21 aromatic protons).

C8 Linker ^1H NMR, CDCl_3 , Major identifying signals were 1.12 (CH_3 of reduced L-threonine), 3.80 (2 CH_2 of DMT), 5.42 (CH_2 of FMOC), 6.18 and 6.321 (2 NH), 6.82-7.80 (21 aromatic protons).

C12 Linker ^1H NMR, CDCl_3 , major identifying signals were 1.12 (CH_3 of reduced L-threonine), 3.78 (2 CH_2 of DMT), 4.59 (CH_2 of FMOC), 6.8-7.8 (21 aromatic protons).

C10 Linker ^1H NMR, CDCl_3 , 1.18 (CH_3 of reduced L-threonine), 3.78 (2 CH_2 of DMT), 4.40 (CH_2 of FMOC), 6.8-7.8 (21 aromatic protons) all the CH_2 and CH (non-aromatics were also accounted for but not assigned).

EXAMPLE 7

METHYLPHOSPHINYLACTION OF THE SECONDARY HYDROXYL MOIETY OF THE NON-NUCLEOTIDE REAGENTS

A DMT blocked linker arm made according to the procedure described in Example 6 above (4 mmol) was dried by co-evaporation with dry pyridine and the residue was dissolved in 20 ml of anhydrous dichloromethane. Under closed argon atmosphere

1.5 equivalent of diisopropylethylamine was added and 1.2 equivalent of N,N-diisopropylmethyphosphinamidic chloride $[(CH_3)_2CH]_2NP(CH_3)Cl$ was added dropwise. The reaction was completed in 45 min. The solvent was removed under reduced pressure and the residue was purified on a flash Silica Gel column. The column was packed with ethyl acetate/hexane (1:) containing 5% triethylamine and washed with the ethyl acetate/hexane containing 1% triethylamine. The reaction mixture was then loaded on the column and the product was eluted with ethyl acetate/hexane (1:1) containing 1% triethylamine.

Other non-nucleotide reagents are prepared by coupling of the linker arm-modified reagents made according to the methods described in Example 6 with other phosphorylating agents such as N,N-diisopropylmethyl phosphonamidic chloride, $[(CH_3)_2CH]_2NP(OCH_3)Cl$, and 2-cyano-ethyl N,N-diisopropylchlorophosphoramidite, $[(CH_3)_2CH]_2NP(Cl)OCH_2CH_2CN$. Such reagents are useful in the synthesis of phosphate diester coupled non-nucleotide-oligomers.

CQ 1H NMR, $CDCl_3$, 0.9-1.3 (18 protons of 6 CH_3 's), 3.11 (CH_2 of FMOC), 3.78 (2 CH_3 's of DMT), 4.42 (CH_2 -O-DMT), 4.98 (NH), 6.8-7.8 (21 aromatic protons).

C4 1H NMR, $CDCl_3$, 0.9-1.2 (18 protons of 6 CH_3 's), 1.88 (CH_2), 2.21 (CH_2), 3.08 (CH_2 of FMOC), 3.80 (2 CH_3 's of DMT), 4.36 (CH_2 -O-DMT), 5.16 (NH), 5.75 (NH), 6.8-7.8 (21 aromatic protons).

C6 1H NMR, $CDCl_3$, 0.9-1.2 (18 protons of 6 CH_3 's), 1.18-2.2 (4 CH_2 's), 3.07 (CH_2 of FMOC), 3.78 (2 CH_3 's of DMT), 4.42 (CH_2 -O-DMT), 5.6 and 6.21 (2 NH), 6.8-7.8 (21 aromatic protons).

EXAMPLE 8

METHYLPHOSPHINYLACTION OF THE SECONDARY HYDROXY MOIETY OF A NON-NUCLEOTIDE REAGENT HAVING A C6-LINKER ARM

A 4 mmol portion of a dimethoxytrityl(DMT)-blocked non-nucleotide reagent having a C6 linker arm (prepared according to

the methods described in Example 6 herein) was dried by co-evaporation with dry pyridine. The residue was dissolved in 20 ml of anhydrous dichloromethane. Under a closed argon atmosphere, 1.5 equivalents of N,N-diisopropylethylamine was added; then 1.2 equivalent of N,N-diisopropylmethylphosphoramidic chloride $[(CH_3)_2CH]_2NP(Cl)OCH_3$ was added dropwise. The reaction mixture was then worked up using the procedures described in Example 7 to give 3.2 mM of the above-identified product.

1H NMR in $CDCl_3$, δ ppm: 1-1.5 (5 methyl and 1 methylene), 1.42 (CH_2), 1.73 and 1.73 (2 CH_2), 2.21 (CH_2-N), 3.15 ($CH_2-C=O$), 3.78 (2 CH_3 of DMT), 6.80-7.85 (21 aromatic protons). Other proton signals present were not assigned.

EXAMPLE 9

PREPARATION OF A PHOSPHATE DIESTER OLIGOMER WHICH INCORPORATES A METHOXYPHOSPHORAMIDITE NON-NUCLEOTIDE REAGENT HAVING A C8 LINKER ARM

A phosphate diester oligodeoxyribonucleotide was synthesized which incorporated a C8 methoxyphosphoramidite non-nucleotide reagent in the following sequence:

5'-TTT-AAG-CAG-AGT-TCA-AAA-GCC TT-CAG-CG-(C8-Linker)-T-3'

was prepared according to the following procedure.

The C8 methoxyphosphoramidite non-nucleotide reagent (1-Q-dimethoxytrityl-2-N[N'-(N"-fluorenyl-methoxycarbonyl-6-aminohexanoyl)-2-aminoacetyl]-3-Q- N-diisopropylmethoxyphosphinyl]-2-amino-1,2-dihydroxybutane) was dissolved in dry acetonitrile at a concentration of 100 mM and coupled into the oligonucleotide sequence using a Biossearch Model 8750 DNA synthesizer by standard phosphoramidite chemistry (M.H. Caruthers, et al., Methods of Enzymology 14:287-313 (1985)) according to the manufacturer's recommendations. The

5'-dimethoxytrityl protecting group was left on at the end of the synthesis to permit purification on a Sep-PakTM C18 cartridge (Millipore/Waters, Bedford, MA) as described by K.M. Lo et al. (1984, Proc. Natl. Acad. Sci. USA, 81, pp. 2285-2289). During this procedure, the dimethoxytrityl protecting group was removed.

EXAMPLE 10

PREPARATION OF METHYLPHOSPHONATE OLIGOMERS WHICH INCORPORATE NON-NUCLEOTIDE REAGENTS

(a) Preparation of Methylphosphonate Oligomers

Methylphosphonate oligomers which incorporated non-nucleotide reagents of the present invention were synthesized using methylphosphonamidite monomers and non-nucleotide methylphosphonamidite non-nucleotide reagents, according to chemical methods described by P.S. Miller et al. (1983, Nucleic Acids Res., 11, pp. 6225-6242), A. Jager and J. Engels (1984, Tetrahedron Lett., 25, pp. 1437-1440), and M.A. Dorman et al. (1984, Tetrahedron, 40, pp. 95-102). Solid-phase synthesis was performed on a Bioscience Model 8750 DNA Synthesizer according to the manufacturer's recommendations with the following modifications: "G" and "C" monomers were dissolved in 1:1 acetonitrile/dichloromethane at a concentration of 100 mM. "A" and "T" monomers were dissolved in acetonitrile at a concentration of 100 mM. Non-nucleotide linker reagents were dissolved in acetonitrile at a concentration of 120 mM. DEBLOCK reagent = 2.5% dichloroacetic acid in dichloromethane. OXIDIZER reagent = 25 g/L iodine in 2.5% water, 25% 2,6-lutidine, 72.5% tetrahydrofuran. CAP A = 10% acetic anhydride in acetonitrile. CAP B = 0.625% N,N-dimethylaminopyridine in pyridine. The 5'-dimethoxytrityl protecting group was left on at the end of the synthesis to facilitate purification of the oligomers, as described below.

The crude, protected non-nucleotide reagent incorporating methylphosphonate oligomers were removed from the solid support by mixing with concentrated ammonium hydroxide for two hours at room temperature. The solution was drained from the support using an Econo-ColumnTM (Bio-Rad, Richmond, CA) and the support was washed five times with 1:1 acetonitrile/water. The eluted oligomer was then evaporated to dryness under vacuum at room temperature. Next, the protecting groups were removed from the bases with a solution of ethylenediamine/ethanol/acetonitrile/water (50:23.5:23.5:2.5) for 6 hours at room temperature. The resulting solutions were then evaporated to dryness under vacuum.

(b) Purification of linker-modified methylphosphonate oligomers.

The 5'-dimethoxytrityl (trityl) containing oligomers were purified from non-tritylated failure sequences using a Sep-PakTM C18 cartridge (Millipore/Waters, Bedford, MA) as follows: The cartridge was washed with acetonitrile, 50% acetonitrile in 100 mM triethylammonium bicarbonate (TEAB, pH 7.5), and 25 mM TEAB. Next, the crude methylphosphonate oligomer was dissolved in a small volume of 1:1 acetonitrile/water and then diluted with 25 mM TEAB to a final concentration of 5% acetonitrile. This solution was then passed through the cartridge. Next, the cartridge was washed with 15-20% acetonitrile in 25 mM TEAB to elute failure sequences from the cartridge. The trityl-on oligomer remaining bound to the cartridge was then detritylated by washing with 25 mM TEAB, 2% trifluoroacetic acid, and 25 mM TEAB, in that order. Finally, the trityl-selected oligomer was eluted from the cartridge with 50% acetonitrile/water and evaporated to dryness under vacuum at room temperature.

The linker-modified methylphosphonate oligomers obtained from the previous step, above, were further purified by reverse-phase HPLC chromatography as follows: A Beckman System

Gold HPLC, described in a previous example, was used with a Hamilton PRP-1 column (Reno, NV, 10 μ , 7 mm i.d. x 305 mm long). Buffer A = 50 mM triethylammonium acetate (pH 7); Buffer B = 50% acetonitrile in 50 mM triethylammonium acetate (pH 7). The sample, dissolved in a small volume of 10-50% acetonitrile/water, was loaded onto the column while flowing at 2.5-3 ml/minute with 100% Buffer A. Next, a linear gradient of 0-70% Buffer B was run over 30-50 minutes at a flow rate of 2.5-3 ml/minute. Fractions containing full-length non-nucleotide reagent incorporating methylphosphonate oligomer were evaporated under vacuum and resuspended in 50% acetonitrile/water.

EXAMPLE 11

LABELLING OF PHOSPHATE DIESTER OLIGOMERS INCORPORATING A CB LINKER ARM NON-NUCLEOTIDE MONOMER WITH BIOTIN

The phosphate diester oligonucleotide of Example 9 (19 nmoles) was suspended in 115 μ l of 0.15 M HEPES buffer (pH 8.0). Next, NHS-LC-biotin (Pierce Chemical Co., Rockford, IL) was added (10 μ l of a 100 mM solution in dimethylsulfoxide). The solution was heated at 37°C for 30 minutes. Additional NHS-LC-biotin solution was added (10 μ l) and the reaction was continued for 30 minutes at 37°C. Next, the biotinylated oligonucleotide was precipitated by addition of 3 M sodium acetate buffer (pH 5.5, 15 μ l) and absolute ethanol (500 μ l); the resulting solution was chilled in dry ice for 30 minutes. The product was recovered by centrifugation for 15 minutes at 4°C in a microcentrifuge and the supernatant was discarded.

The product was then dissolved in water (100 μ l) and purified by reverse-phase HPLC chromatography according to the following method. The HPLC apparatus consisted of a Beckman System Gold Model 126 Solvent Module and Model 167 Detector interfaced to an IBM compatible computer. A PLRP-S column was used (Polymer Laboratories, 8 μ , 300 Å pore size, 4.6 mm i.d. x

250 mm long). Buffer A = 50 mM triethylammonium acetate (pH 7); Buffer B = 50% acetonitrile in 50 mM triethylammonium acetate (pH 7). A linear gradient of 20-60% B was run over 30 minutes at a flow rate of 1.5 ml/minute. Under these conditions, the biotinylated product oligonucleotide eluted at 10.3 minutes.

EXAMPLE 12

BINDING OF A BIOTINYLATED CE NON-NUCLEOTIDE MONOMER MODIFIED PHOSPHATE DIESTER OLIGONUCLEOTIDE TO A STREPTAVIDIN-MODIFIED SOLID SUPPORT

The biotinylated oligonucleotide of Example 11 was labelled with ^{32}P at the 5'-terminus using (γ - ^{32}P)-ATP (3000 Ci/mmol) and T4 polynucleotide kinase as follows: 10 pmol of the oligonucleotide was dissolved in 10 μl of 50 mM Tris (pH 7.8), 10 mM MgCl_2 , 5 mM DTT, 0.1 mM EDTA, 0.1 mM spermidine containing 50 μCi of (γ - ^{32}P)-ATP. T4 polynucleotide kinase (4 units) was added, and the solution was incubated for 90 minutes at room temperature. The radiolabeled product was purified on a NensorbTM-20 column (New England Nuclear/DuPont) according to the manufacturer's instructions.

The ^{32}P -labelled biotinylated oligonucleotide (10,000 cpm) was dissolved in 0.5 ml of 50 mM sodium phosphate (pH 6.8), 0.5 M sodium chloride, 2 mM EDTA in a 1.5 ml microcentrifuge tube. Controls were also prepared containing the same components plus 1 mg/ml biotin (Calbiochem Corp., San Diego, CA). Next, 50 μl of streptavidin-agarose (Pierce Chemical Co., Rockford, IL) was added to each tube and the contents were mixed on a vortexer for 15 minutes. The tubes were then centrifuged for 2 minutes in a microcentrifuge to pellet the streptavidin-agarose; and the supernatants were transferred to fresh tubes. The pellets were then washed twice with 0.5 ml of buffer (See above) and the washes were likewise separated by centrifugation and transferred to fresh tubes. The tubes were counted for radioactivity in a

scintillation counter. Samples prepared without the addition of free biotin bound to the streptavidin-agarose support at greater than 85%. Samples prepared with added free biotin (controls) bound to the support at less than 0.5%.

EXAMPLE 11

LABELLING OF A METHYLPHOSPHONATE OLIGOMER INCORPORATING A C4 LINKER NON-NUCLEOTIDE MONOMER WITH PSORALEN

A C4 linker-modified methylphosphonate oligomer was prepared having the following sequence:

5'GGC-TTT-TGA-(C4-linker)-ACT-CTG-CTT-3'

where the bold letters (including C4-linker) refer to bases or non-nucleotide monomeric units connected by methylphosphonate linkages and the upper case letters refer to bases connected by diester linkages. The method of synthesis and purification of this oligonucleotide is described in a previous example, above.

This oligomer was labelled with a psoralen-NHS labelling reagent as follows:

The following coupling reaction of NHS-psoralen reagent to linker arm (present in the oligomer) was carried out in a 1.5 ml polypropylene microfuge tube. Approximately 3.4 mg (98 OD₂₆₀ units) of the oligomer was dissolved in 100 µl of 1:1 acetonitrile/water. Next, the following reagents were added in order, with vortexing at each addition to avoid precipitation of the oligomer: dimethylsulfoxide (170 µl), water (100 µl), 1 M HEPES buffer, pH 8.0 (50 µl), and 50 mM psoralen-NHS reagent in dimethylsulfoxide (80 µl). Total volume: 500 µl. The mixture was reacted for 2.5 hours at room temperature in the absence of light. Ethanol (1 ml) was then added, and the resulting solution was chilled at -20°C overnight. The tube was then spun in a microcentrifuge for 5 minutes and the supernatant was aspirated

and discarded. The resulting pellet was resuspended in 500 μ l of 1:1 acetonitrile/water and filtered through a 0.22 μ Durapore™ membrane to remove particulate material.

HPLC purification of the solution of crude psoralen-oligomer conjugate described above was conducted as follows: A Beckman System Gold analytical HPLC system was used with a Hamilton PRP-1 column (4.1 x 250 mm). Buffers used for elution were: Buffer A - 50 mM triethylammonium acetate (pH 7); Buffer B - 50% acetonitrile in 50 mM triethylammonium acetate (pH 7). The sample was loaded onto the column in five 100 μ l portions at two minute intervals with a 500 μ l sample loop while the column was flowing at 1.5 ml/min with 10% Buffer B. Next, a linear gradient from 10 - 70% Buffer B was run over 30 minutes. Fractions were collected at 0.5 minute intervals. Under these conditions, unmodified oligomer and psoralen-modified oligomer eluted at 17.9 minutes and 21.7 minutes, respectively. Fractions containing the psoralen-modified oligomer were pooled and evaporated. The overall yield was 16%.

EXAMPLE 14

CROSS-LINKING OF PSORALEN-LABELLED METHYLPHOSPHONATE OLIGOMER TO A COMPLEMENTARY PHOSPHATE DIESTER OLIGONUCLEOTIDE TARGET

A phosphate diester oligonucleotide complementary to the methylphosphonate oligomer of Example 13 was synthesized on a Biosearch Model 8750 DNA Synthesizer according to the manufacturer's recommendations; this oligonucleotide has the following sequence:

5'-AAG-CAG-AGT-TCA-AAA-GCC-3'

The psoralen-modified methylphosphonate oligomer, prepared according to the procedure of Example 13, was labeled at its 5'-end with 32 P as described above (See Example 12). The 32 P-

labeled, psoralen modified oligomer (10-50,000 cpm) was hybridized to its diester oligonucleotide target (1 pmol, above), in a borosilicate glass vial containing 10 μ l of 10 mM Tris (pH 7.2), 0.1 mM EDTA, 0.03% potassium sarkosylate at 37°C for 30 minutes. Control reactions were also run without the diester oligonucleotide target. The vials were then irradiated at 365 nm on crushed ice using a Model B-100A long wavelength ultraviolet lamp (UVP, Inc., San Gabriel, CA) at a distance of 15 centimeters. Intensity of irradiation at this distance was greater than or equal to 60 μ W/100 cm². After 30 minutes, 90% formamide containing 0.1% bromphenol blue, 0.1 M tris-borate-EDTA buffer (pH 8.2) was added (5 μ l) and the samples were loaded onto a 15% polyacrylamide gel containing 7 M urea (0.5 mm). The gel was electrophoresed at 900 V for 2 hours. The wet gel was then placed between two sheets of Saran WrapTM and exposed to XAR-5 film (Eastman-Kodak, Rochester, NY) for 30-60 minutes. The resulting autoradiograph revealed upper bands for samples containing diester target oligonucleotide which migrated slower in the gel than the bands corresponding to psoralen-modified methylphosphonate oligomer alone. Using the autoradiograph as a template, the bands were then excised from the wet gel with a scalpel and counted in a scintillation counter. Based on this method, it was determined that cross-linking of psoralen-modified methylphosphonate oligomer to its complementary diester target was greater than 95%.

Claims

We claim:

1. A chirally pure non-nucleotide reagent suitable for preparing a nucleotide/non-nucleotide polymer and which remains chirally pure when incorporated into said polymer which comprises a non-nucleotide monomeric unit which has a chirally pure non-nucleotide skeleton and connected to the skeleton has a ligand moiety and first and second coupling groups, wherein the first coupling group is capable of coupling the skeleton to a first additional monomeric unit, while the second coupling group remains inactivated so as to be substantially incapable of coupling, but which can thereafter be activated under non-adverse conditions to couple the skeleton to a second additional monomeric unit, wherein said nucleotide/non-nucleotide polymer comprises at least one nucleotide monomeric unit.

2. A non-nucleotide reagent according to claim 1 wherein at least one of the first and second additional monomeric units comprises a nucleotide monomeric unit.

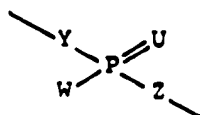
3. A non-nucleotide reagent according to claim 1 wherein said nucleotide/non-nucleotide polymer comprises at least one phosphate diester linkage between monomeric units.

4. A non-nucleotide reagent according to claim 3 wherein said nucleotide/non-nucleotide polymer comprises a phosphate diester oligonucleotide.

5. A non-nucleotide reagent according to claim 1 wherein said nucleotide/non-nucleotide polymer comprises at least one linkage between monomeric units selected from phosphorothioate, phosphoramidate and neutral phosphate ester linkages.

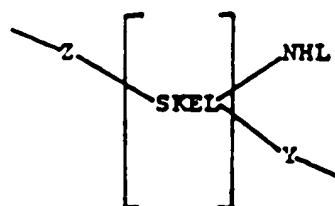
6. A non-nucleotide reagent according to claim 1 wherein said nucleotide/non-nucleotide polymer comprises linkages between monomeric units of the following formula:

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wherein U is oxygen, sulfur or imino; Y is $-\text{CH}_2-$, $-\text{S}-$, $-\text{O}-$, or $-\text{NH}-$; Z is $-\text{S}-$, $-\text{O}-$ or $-\text{NH}-$; and W is alkyl, aryl, alkoxy, aryloxy, alkylthio, arylthio, S' , O' , amino or substituted amino.

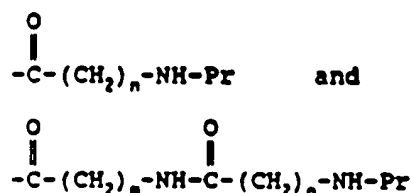
7. A chirally pure non-nucleotide reagent according to claim 1 wherein said non-nucleotide monomeric unit comprises:



wherein SKEL comprises a chirally pure non-nucleotide skeleton of about 1 to about 20 carbon atoms, wherein $-\text{NHL}$, Y, and Z are covalently linked to a carbon atom of SKEL; L is a ligand; Y is $-\text{CH}_2-$, $-\text{O}-$, $-\text{S}-$ or $-\text{NH}-$; and Z is $-\text{O}-$, $-\text{S}-$ or $-\text{NH}-$.

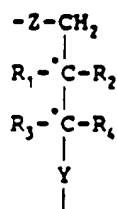
8. A non-nucleotide reagent according to claim 7 wherein SKEL comprises a backbone of about 1 to about 10 carbon atoms between Y and Z.

9. A non-nucleotide reagent according to claim 8 wherein L comprises $-\text{Pr}$ or a protected linker arm selected from:



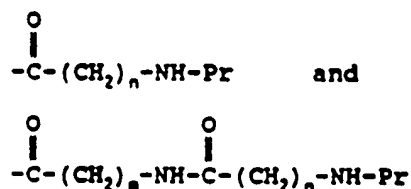
wherein n and m are independently integers from about 1 to about 15 and Pr is a protecting group removable under non-adverse conditions.

10. A non-nucleotide reagent according to claim 1 wherein said non-nucleotide monomeric unit comprises:



wherein at least one of \dot{C} is a carbon atom which comprises a chiral center, one of R_1 and R_2 is hydrogen and the other is $-NH-L$ wherein L is a ligand; one of R_3 and R_4 is hydrogen and the other is lower alkyl of about 1 to about 10 carbon atoms; Y is $-CH_2-$, $-O-$, $-S-$ or $-NH-$; and Z is $-O-$, $-S-$, or $-NH-$.

11. A non-nucleotide reagent according to claim 10 wherein the L comprises $-Pr$ or a protected linker arm selected from:



wherein n and m are independently integers from about 1 to about 15 and Pr is a protecting group removable under non-adverse conditions.

12. A non-nucleotide reagent according to claim 11 wherein said nucleotide/non-nucleotide polymer comprises at least one phosphate diester linkage between monomeric units.

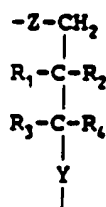
13. A non-nucleotide reagent suitable for preparing a nucleotide/non-nucleotide polymer having at least one alkyl- or aryl-phosphonate linkage between monomeric units which comprises a non-nucleotide monomeric unit which has a non-nucleotide skeleton and connected to the skeleton has a ligand moiety and first and second coupling groups, wherein the first coupling group is capable of forming an alkyl- or aryl-phosphonate linkage between the skeleton and a first additional monomeric unit, while

the second coupling group remains inactivated so as to be substantially incapable of coupling but which can thereafter be activated under non-adverse conditions to couple the skeleton to a second additional monomeric unit, wherein said nucleotide/nucleotide polymer comprises at least one nucleotide monomeric unit.

14. A non-nucleotide reagent according to claim 13 wherein at least one of the first and second additional monomeric units comprises a nucleotide monomeric unit.

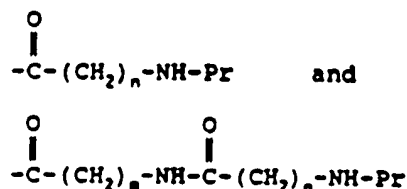
15. A non-nucleotide reagent according to claim 13 wherein said alkyl- or aryl-phosphonate linkage comprises a methylphosphonate linkage.

16. A non-nucleotide reagent according to claim 15 wherein said monomeric units comprises:



wherein one of R_1 and R_2 is hydrogen and the other is $-NH-L$ wherein L is a ligand; one of R_3 and R_4 is hydrogen and the other is lower alkyl of about 1 to about 10 carbon atoms; Y is $-CH_2-$, $-O-$, $-S-$, or $-NH-$; and Z is $-O-$, $-S-$, or $-NH-$.

A-1 17. A non-nucleotide reagent according to claim 16 wherein L comprises $-Pr$ or a protected linker arm selected from:



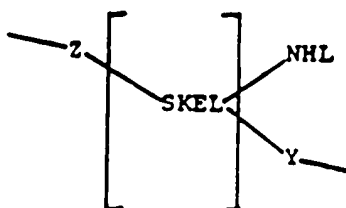
wherein n and m are independently integers from about 1 to about 15 and Pr is a protecting group removable under non-adverse conditions.

19. A non-nucleotide reagent suitable for preparing a nucleotide/non-nucleotide polymer having at least one alkyl- or aryl-phosphonate linkage between monomeric units and which remains chirally pure when incorporated into said polymer which comprises a non-nucleotide monomeric unit having a chirally specific non-nucleotide skeleton and connected to the skeleton; a ligand moiety; and first and second coupling groups wherein the first coupling group is capable of forming an alkyl- or aryl-phosphonate linkage between the chirally pure skeleton and a first additional monomeric unit while the second coupling group remains inactivated so as to be substantially incapable of coupling but which can thereafter be activated under non-adverse conditions to couple the chirally pure skeleton to a second additional monomeric unit, wherein said nucleotide/non-nucleotide polymer comprises at least one nucleotide monomeric unit.

20. A non-nucleotide reagent according to claim 19 wherein at least one of the first and second additional monomeric units comprises a nucleoside unit.

21. A non-nucleotide reagent according to claim 19 wherein said alkyl- or aryl-phosphonate linkage comprises a methylphosphonate linkage.

22. A chirally pure non-nucleotide reagent according to claim 21 wherein said non-nucleotide monomeric unit comprises:

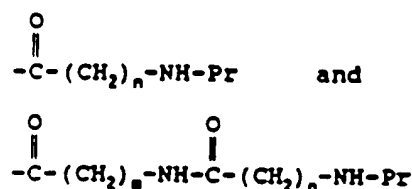


wherein SKEL comprises a chirally pure non-nucleotide skeleton of about 1 to about 20 carbon atoms, wherein -NHL, Y and Z are

covalently linked to a carbon atom of SKEL, L is a ligand, Y is $-\text{CH}_2-$, $-\text{O}-$, $-\text{S}-$ or $-\text{NH}-$ and Z is $-\text{O}-$, $-\text{S}-$ or $-\text{NH}-$.

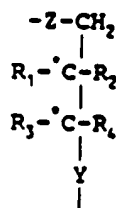
23. A non-nucleotide reagent according to claim 22 wherein SKEL comprises a backbone of about 1 to about 10 carbon atoms between Y and Z.

24. A non-nucleotide reagent according to claim 23 wherein L comprises $-\text{Pr}$ or a protected linker arm selected from:



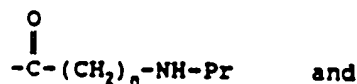
wherein n and m are independently integers from about 1 to about 15 and Pr is a protecting group removable under non-adverse conditions.

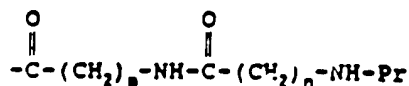
25. A non-nucleotide reagent according to claim 19 wherein said non-nucleotide monomeric units comprises:



wherein at least one of C^* is a carbon which comprises a chiral center, one of R_1 and R_2 is hydrogen and the other is $-\text{NH}-\text{L}$ wherein L is a ligand; one of R_3 and R_4 is hydrogen and the other is lower alkyl of about 1 to about 10 carbon atoms; Y is $-\text{CH}_2-$, $-\text{O}-$, $-\text{S}-$, or $-\text{NH}-$; and Z is $-\text{O}-$, $-\text{S}-$, or $-\text{NH}-$.

26. A non-nucleotide reagent according to claim 25 wherein L comprises $-\text{Pr}$ or a protected linker arm selected from:

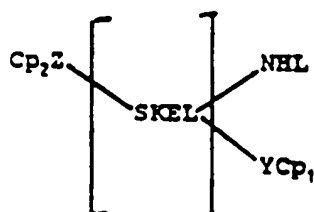




wherein n and m are independently integers from about 1 to about 15 and Pr is a protecting group removable under non-adverse conditions.

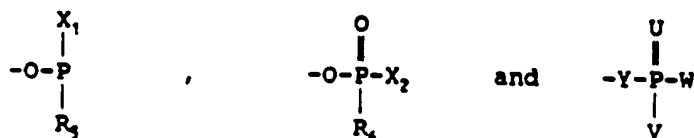
27. A non-nucleotide reagent according to claim 26 wherein said alkyl- or aryl-phosphonate linkage comprises a methylphosphonate linkage.

28. A chirally pure non-nucleotide reagent of the formula:



wherein SKEL comprises a chirally pure non-nucleotide skeleton of about 1 to about 20 carbon atoms, wherein -NHL, Y and Z are covalently linked to a carbon atom of SKEL, L is a ligand, -YCp₁ is a first coupling group and -ZCp₂ is a blocked second coupling group; where Y is -CH₂-, -O-, -S-, or -NH-; and Z is -O-, -S-, or -NH-.

29. A non-nucleotide reagent according to claim 28 wherein the first coupling group, -YCp₁, is selected from

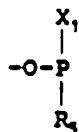


wherein X₁ is halogen or substituted amino; X₂ is halogen, substituted amino or O'; R₃ is alkyl, optionally substituted alkoxy or optionally substituted aryloxy; and R₄ is alkyl, optionally substituted alkoxy or optionally substituted aryloxy; or, if X₂ is O', optionally hydrogen; U is oxygen, sulfur or

amino, V is O', S', or substituted amino; and W is alkyl, aryl, alkoxy, aryloxy, alkylthio, arylthio, S', O', amino or substituted amino.

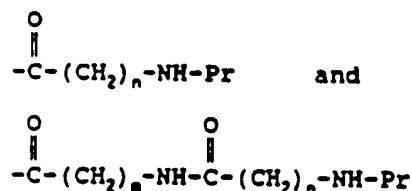
30. A non-nucleotide reagent according to claim 29 wherein the blocked second coupling group, $-2Cp_2$, has a protecting group cleavable under non-adverse deblocking conditions to recover the second coupling group, $-2H$.

31. A non-nucleotide reagent according to claim 30 wherein the first coupling group is



wherein R_2 is methyl and X_1 is diisopropylamino.

32. A non-nucleotide reagent according to claim 31 wherein said ligand comprises $-Pr$ or a protected linker arm selected from:

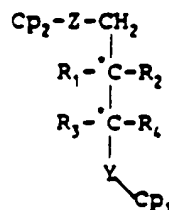


wherein n and m are independently integers from about 1 to about 15 and Pr is a protecting group removable under non-adverse conditions.

33. A non-nucleotide reagent according to claim 32 wherein n and m are independently integers between 1 and 5.

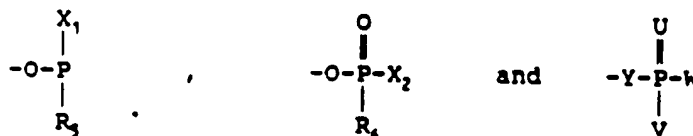
34. A non-nucleotide reagent according to claim 33 wherein Z is oxygen.

35. A chirally pure non-nucleotide reagent of the formula:



wherein 'C' is a carbon atom which comprises a chiral center, wherein one of R_1 and R_2 is hydrogen and the other is -NH-L where L is a ligand moiety; one of R_3 and R_4 is hydrogen and the other is lower alkyl of about 1 to about 10 carbon atoms, -YCp_1 is a first coupling group and -ZCp_2 is a blocked second coupling group; where Y is $\text{-CH}_2\text{-}$, -O- , -S- or -NH- ; and Z is -O- , -S- , or -NH- .

36. A non-nucleotide reagent according to claim 35 wherein the first coupling group, -YCp_1 , is selected from

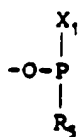


wherein X_1 is halogen or substituted amino; X_2 is halogen, substituted amino or O^+ ; R_3 is alkyl, optionally substituted alkoxy or optionally substituted aryloxy; and R_4 is alkyl, optionally substituted alkoxy or optionally substituted aryloxy; or, if X_2 is O^+ , optionally hydrogen; U is oxygen, sulfur or imino, V is O^+ , S^+ , or substituted amino; and W is alkyl, aryl, alkoxy, aryloxy, alkylthio, arylthio, S^+ , O^+ , amino or substituted amino.

37. A non-nucleotide reagent according to claim 36 wherein the blocked second coupling group, -ZCp_2 , has a protecting group cleavable under non-adverse deblocking conditions to recover the second coupling group, -ZH .

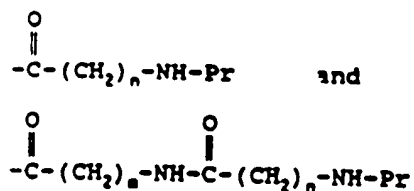
38. A non-nucleotide reagent according to claim 37 wherein said protecting group is a dimethoxytrityl group.

39. A non-nucleotide reagent according to claim 37 wherein the first coupling group is



wherein R_2 is methyl and X_1 is diisopropylamino.

40. A non-nucleotide reagent according to claim 39 wherein said ligand comprises -Pr or a protected linker arm selected from:

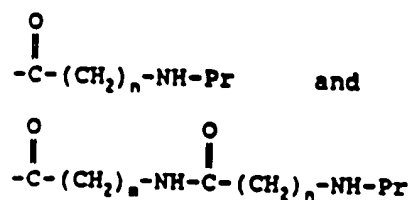


wherein n and m are independently integers from about 1 to about 15 and Pr is a protecting group removable under non-adverse conditions.

41. A non-nucleotide reagent according to claim 40 wherein n and m are independently integers between 1 and 5.

42. A non-nucleotide reagent according to claim 40 wherein Z is oxygen.

43. A non-nucleotide reagent according to claim 35 wherein said ligand comprises -Pr or a protected linker arm selected from:



wherein n and m are independently integers between 1 and 15 and Pr is a protecting group removable under non-adverse conditions.

44. A chirally pure non-nucleotide reagent suitable for preparing a nucleotide/non-nucleotide polymer and which remains chirally pure when incorporated into said polymer which comprises a non-nucleotide monomeric unit which has a chirally pure non-nucleotide skeleton and connected to the skeleton has a ligand moiety and first and second coupling groups, wherein the first coupling group couples the skeleton to a support by a linkage which may be cleaved under non-adverse conditions, while the second coupling group remains inactive, but which can thereafter be activated under non-adverse conditions to couple the skeleton to an additional monomeric unit, wherein said nucleotide/non-nucleotide polymer comprises a nucleotide monomeric unit.

45. A non-nucleotide reagent according to claim 44 wherein said skeleton is coupled to said support by an ester linkage.

46. A non-nucleotide reagent according to claim 44 wherein said nucleotide/non-nucleotide polymer comprises at least one alkyl- or aryl-phosphonate linkage between monomeric units.

47. A non-nucleotide reagent suitable for preparing a nucleotide/non-nucleotide polymer having at least one alkyl- or aryl-phosphonate linkage between monomeric units which comprises a non-nucleotide monomeric unit which has a non-nucleotide skeleton and connected to the skeleton has a ligand moiety and first and second coupling groups, wherein the first coupling group couples the skeleton to a support by a linkage which may be cleaved under non-adverse conditions, while the second coupling group remains inactive, but which can thereafter be activated under non-adverse conditions to couple the skeleton to an additional monomeric unit, wherein said nucleotide/non-nucleotide polymer comprises a nucleotide monomeric unit.

48. A non-nucleotide reagent according to claim 47 wherein said skeleton is coupled to said support by an ester linkage.

49. An oligomer which comprises at least about 8 monomeric units wherein at least one monomeric unit comprises a non-nucleotide monomeric unit as defined in claim 1.

50. An oligomer according to claim 49 which comprises from about 1 to about 5 independently selected non-nucleotide monomeric units.

51. An oligomer which comprises at least about 8 monomeric units wherein at least one monomeric unit comprises a non-nucleotide monomeric unit as defined in claim 13.

52. An oligomer according to claim 51 which comprises a methylphosphonate oligomer.

53. An oligomer which comprises at least about 8 monomeric units wherein at least one monomeric unit comprises a non-nucleotide monomeric unit as defined in claim 19.

54. An oligomer according to claim 53 which comprises a methylphosphonate oligomer.

55. An oligomer according to claim 54 which comprises from about 1 to about 5 independently selected non-nucleotide monomeric units.

56. An oligomer which comprises at least about 8 monomeric units wherein at least one monomeric unit comprises a non-nucleotide monomeric unit according to claim 23.

57. An oligomer according to claim 56 which comprises a methyl phosphonate oligomer.

58. An oligomer according to claim 57 which comprises from about 1 to about 5 independently selected non-nucleotide monomeric units.

59. An oligomer according to claim 58 where n and m are independently integers from 1 to 5.

60. An oligomer which comprises at least 8 monomeric units wherein at least one monomeric unit comprises a non-nucleotide monomeric unit according to claim 26.

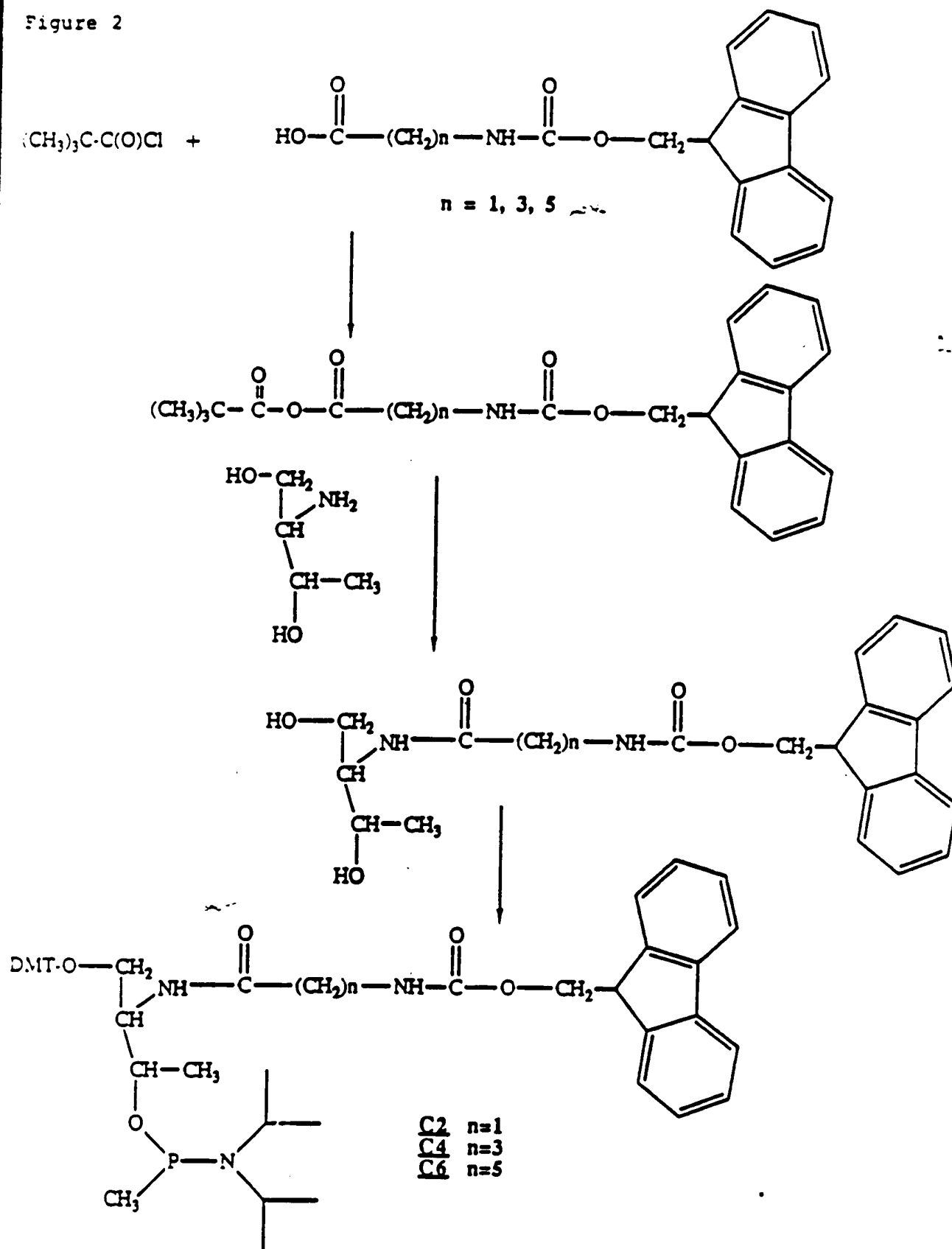
61. An oligomer according to claim 60 which comprises a methylphosphonate oligomer.

62. An oligomer according to claim 61 which comprises from about 1 to about 5 independently selected non-nucleotide monomeric units.

63. An oligomer according to claim 62 wherein n and m are independently integers from 1 to 5.

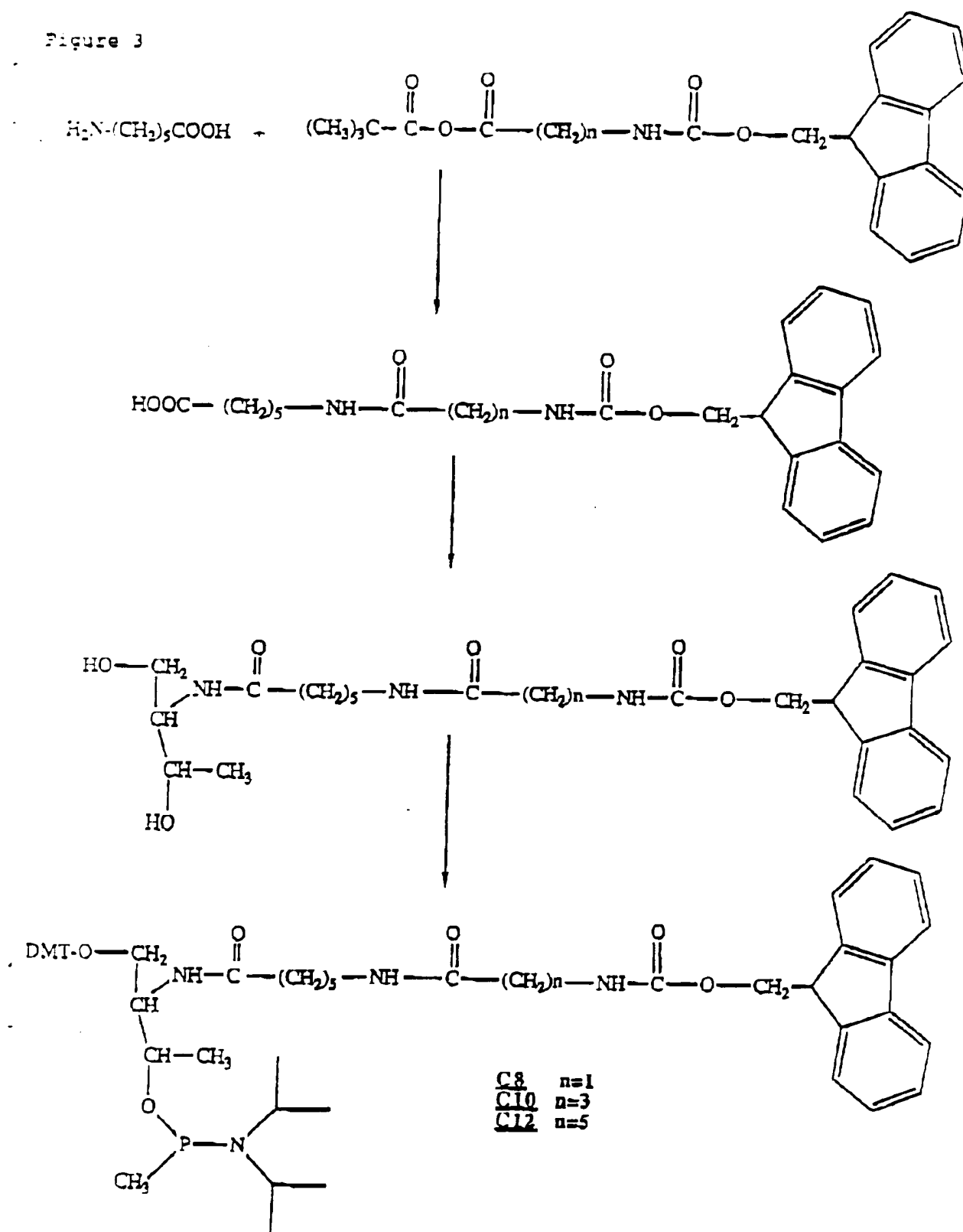
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Figure 2



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Figure 3



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Figure 4

